Characterization of a Novel Necrotic Response of *Glycine max* Line 'PI96188' to *Xanthomonas axonopodis* pv. *glycines*

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Typical susceptible symptoms of the bacterial pustule disease caused by *Xanthomonas axonopodis* pv. *glycines* are pustule and chlorotic haloes that usually occur in leaves of *Glycine max* plants. The soybean genotype 'PI96188' showed an atypical response to all tested races *X. axonopodis* pv. *glycines*, accompanied with necrosis without chlorotic haloes on the underside of the necrotic symptoms. *X. axonopodis* pv. *glycines* 8ra grew to levels from 10 to 100 fold lower on PI96188 than on susceptible cultivar Jinjool, but 10-fold higher than on the resistant cultivar CNS. The chlorophyll content in PI96188 leaves remained unchanged until 12 days after bacterial infection. Ultrastructural observation showed that the infected leaf cells of PI96188 had intact normal chloroplasts compared to those of the susceptible cultivar Jinjool. Chloroplast degradation or the absence of chloroplasts in cells of the infected tissues was observed in Jinjool. Senescence-related *ACS7* gene was significantly induced in PI96188 compared to those in Jinjool at 2 days after inoculation. While photosynthesis-related *rbcS* gene showed the dramatic change in Jinjool, this gene was constitutively expressed in PI96188. However, expression of the defense-related genes, such as peroxidase and isoflavone synthase in the infected PI96188 leaves was similar to that in Jinjool. Together, these results suggest that the novel necrotic symptom in PI96188 is a kind of resistant response different from a typical hypersensitive response in the resistant genotypes.

Keywords : bacterial pustule disease, defense gene, resistance, soybean

Soybean (*Glycine max* (L) Merr.) is one of the most protein-rich oilseed crops for the leading edible oil source worldwide (Manjaya and Pawer, 1999). Bacterial pustule disease caused by *Xanthomonas axonopodis* pv. *glycines* (Nakano) Dye is one of the destructive diseases in soybean-growing areas of the world where warm weather and frequent showers prevail during the growing season (Sinclair and Backman, 1989). Early symptoms of bacterial pustule disease are minute and pale green spots with elevated centers on either or both leaf surfaces. Later, a small, raised, light-colored pustule forms in the center, usually in lesions on the underleaf surface. A brownish-yellow haloes appear around the edge of pustules. The spots vary from specks to large, irregular, mottled brown areas, which develop when lesions coalesce (Colye, 1989).

Three of several factors that may contribute to the virulence of *X. axonopodis* pv. *glycines* has been suggested to be production of auxin, extracellular polysaccharides, and cellulase (Swing and Civerolo, 1993). Typical symptoms of the bacterial pustule disease in soybean plants are pustule and chlorotic haloes. Pustule symptoms are formed by hypertrophy of parenchyma cells in soybean leaves. Auxin of bacterial origin and tryptophan of plant origin are involved in the development of pustule symptom in soybean (Kim et al., 2001a). Attack by plant pathogenic bacteria frequently causes a chlorotic symptom in plants. The chlorosis that develops upon infection by a number of leaf-spotting xanthomonads and pseudomonads is suggested to form as the result of a direct effect upon chloroplasts, possibly by toxic metabolites of the pathogen (Durbin, 1971; Gross and Cody, 1985). Earlier findings of the induction of chlorotic haloes in plant leaves by the action of toxins produced by *P. syringae* pv. *tabaci* strongly suggested that the toxin may either destroy chlorophyll or inhibit chlorophyll biosynthesis (Braun, 1955). Spectroscopic and chemical analysis of the chlorophyll in toxin-treated leaves revealed that concentrations of chlorophyll in chlorotic tissues were much lower than those in comparable normal tissues (Turner, 1981). The chlorotic haloes of bacterial pustule disease caused by *X. axonopodis* pv. *glycines* are not fully understood. Chlorophyll degradation

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often takes place during the turnover of chlorophyll, but not in a synchronized manner, as in the programmed cell death during the leaf senescence in the autumn. Furthermore, chlorophyll degradation occurs during cell death caused by external factors, such as injuries sustained by low or high temperature, or by pathogen attack (Takamiya et al., 2000). Chlorotic haloes are a macroscopic symptom of chloroplast degradation. Since chloroplast proteins, pigments and lipids are degraded, the structural integrity of the thylakoid network breaks down. Therefore, the capacity for photosynthetic electron transport and CO_2 fixation decreases substantially in plant cells. When chlorosis occurs in the leaves, photosynthesis rate in the infected leaves is remarkably lower than the healthy leaves.

In the earliest study, Hartwig and Lehman (1951) observed a high degree of resistance in soybean cultivar CNS mediated by a single recessive gene, designated rxp. A high field resistance to bacterial pustule disease also was observed in soybean genetic stocks (Fartushnyak et al., 1987). The rxp gene confers partial resistance by increasing the number of bacterial cells necessary for infection rather than by restricting pathogen growth within host tissues. The genotypes having the rxp gene were found to show reduced intensity of symptoms with higher concentrations of inoculum compared to the susceptible genotypes under the controlled environmental conditions (Groth and Braun, 1986). The genetic stock, P-4-2, gave a highly resistant reaction even when inoculated with a high concentration of inoculum (Sharma et al., 1993). A genetic study further revealed that the resistance in P-4-2 was controlled by a duplicate recessive gene action (Manjaya and Pawar, 1999), which indicates that the resistance genes in P-4-2 are different from the rxp gene. In contrast, the genotype, PK472, which was resistant to X. axonopodis pv. glycines in the field (Verma, 1990), was susceptible under controlled conditions. However, the appearance of symptoms in the genotype, PK472, was delayed, compared to those in the susceptible control ones (Sharma et al., 1993).

In the present study, various soybean genotypes have been collected worldwide to evaluate the responses of these soybean genotypes to X. axonopodis pv. glycines. Among the tested genotypes, the disease symptoms of soybean genotype PI96188 were obviously different from those of other genotypes. To investigate whether this novel disease response of the genotype PI96188 is a resistant response, the soybean genotype PI96188 and other comparable soybean cultivars were evaluated with respect to the fitness of X. axonoppodis pv. glycines in these soybean genotypes. The expression of disease symptoms and defense-related genes in those resistant and susceptible soybean genotypes was also examined using the electron microscopy and RNA gel blot analysis.

Materials and Methods

Plant materials and bacterial pathogens. Soybean (*Glycine max* L.) line PI96188, and cultivars Jinjoo1, Suwon157, Danbaekkong, CNS1, and Peking which differ in resistance to bacterial pustule disease caused by X. axonopodis pv. glycines (Nakano) Dye were used in this study. Soybean seeds were germinated on moist filter paper in Petri dishes for 2 days in the dark at 28°C. The germinated seeds were sown in a plastic tray containing soil mix (Oh et al., 1999b). At 15 days after sowing, soybean seedlings were transplanted to pots ($14 \times 12 \times 12$ cm). Soybean plants were cultivated in a greenhouse at 25 ± 2°C, as previously described (Oh, 1997). The strains 8ra, and regional collected strains (SL1017, SL1018, SL1045, SL1157, and SL2098) of X. axonopodis pv. glycines were cultured at 28°C in potato sucrose agar (PSA) (Oh et al., 1999a). Antibiotics were added to the media to give 100 mg/ml rifampicin.

Bacterial inoculation and chemical treatment. To prepare the bacterial inocula for inoculation in soybean leaves, X. axonopodis pv. glycines was cultured on PSA and Pseudomonas syringae pv. syringae strain 61 was cultured on King's B medium at 28°C for 2 days. The bacterial cells were then harvested and suspended in 10 mM $MgCl_2$. Bacterial suspensions were adjusted to 5×10^8 cfu/ml in 10 mM $MgCl_2$ prior to inoculation. Three-week-old soybean plants were inoculated with the bacterial suspension (1×10^8 cfu) by spraying onto the leaf surface.

Measurement of chlorophyll content. To determine chlorophyll content, eight leaf discs (8 mm in diameter) were sampled at random from leaves of PI96188 and Jinjoo1 infected by X. axonopodis pv. gycines 8ra at 3 days intervals. All the extraction experiments were performed in a dark room in order to prevent photo-oxidative loss of chloroplasts. The chlorophyll extracts were homogenized and added with 1 ml of 80% aqueous acetone. After vortexing, samples were centrifuged and green supernatants were taken.

The pellet was re-extracted with 1 ml of 80% aqueous acetone and centrifuged again. The supernatant was added to the supernatant of the first centrifugation to make up to 2 ml. The absorbance of the supernatant was measured at 648 and 664 nm. Chlorophyll content was determined following the formula: $C = 5.24 \times A_{664} + 22.24 \times A_{648}$, where C is the chlorophyll concentration in micrograms per milliliter and A is absorption (Linchtenthaler, 1987).

Electron microscopy. To test the change of organelles of infected regions, the leaves of PI96188 and Jinjoo1 were collected 2 weeks after inoculation. Small pieces (4 mm²)

of the samples were fixed immediately after collection in 1% paraformaldehyde and 0.25% glutaraldehyde in 50 mM cacodylate buffer, pH 7.2, at 4°C overnight. Subsequently, the leaf tissues were rinsed three times with 50 mM cacodylate buffer, pH 7.2 and post-fixed for 2 h with 1% osmic acid at 4°C in the same buffer. After washing three times with 50 mM cacodylate buffer, pH 7.2, dehydration was performed with a graded ethanol series. The tissue samples were embedded in Epon 812. Ultra-thin sections (0.8 μm) from the Epon-embedded material were sliced using a diamond knife, transferred in copper grids and stained with uranyl acetate and Reynolds lead citrate (Lee et al., 2001). All micrographs were taken with LEO 912 AB transmission electron microscope. For each plant, an average of seven samples from three different leaves was investigated. For each sample, 25-30 ultra-thin sections were examined under the electron microscope.

Measurement of bacterial growth in plants. *X. axonopodis* pv. *glycines* 8ra was harvested after incubation for 2 days at 28°C. Bacterial suspensions were adjusted to 5×10^8 cfu/ml in 10 mM $MgCl_2$. Bacterial inoculum was sprayed on the first, fully expanded, trifoliate soybean leaves of 3 weeks old plants using an atomizer. Three leaves taken at one day intervals after inoculation were ground in a microtube containing 1 ml of 10 mM $MgCl_2$. Each sample was serial diluted in 10×10 mM $MgCl_2$. Six droplets of 10 μl samples were smeared onto a LB medium. After incubation for 36-48 h at 28°C, discrete colonies appearing on the LB medium were counted. After bacterial cells were counted, 'cfu/g of fresh weight was used as comparison.

RNA gel blot analysis. Total RNA was extracted from 1.5 g soybean leaf tissues from PI96188 and Jinjoo1 at 0, 2, 4, 6, 12, 24, 48, 72, 96, 120, 144, and 168 hr after inoculation using TRIzole (Gibco BRL, Basel, Switzerland) following the manufacturer's protocol. Extracted RNAs (20 μg) were denatured by heating at 70°C for 10 min in RNA loading dye (Ambion, Austin, Texas, USA) with ethidium bromide, followed by electrophoresis on 12% formaldehyde gels and blotting to Hybond N+ membranes (Amersham Biosciences, Uppsala, Sweden). The *EcoRI/XhoI* fragments of the soybean peroxidase (POX) gene in were cloned pBluescript SK(-) from soybean cDNA library (Yi and Hwang, 1998; Kim et al., 2000). The *EcoRI* fragments of the soybean chalcone synthase (CHS), isoflavone synthase (*IFS*), 1-aminocyclopropan carboxylic acid synthases (*ACS*) 1 and 7, and ribulose-1,5-bisphosphate carboxylase (RUBISCO) small subunit genes were also cloned in pGEM-T from a soybean cDNA library. These genes were ^{32}P-labeled with a random priming kit (Amersham) to use as probes. Hybridization was performed overnight at 65°C

in 5% dextran sulfate, 0.25 M disodium phosphate (pH 7.2), 7% (w/v) SDS and 1 mM EDTA. After hybridization, the filter was washed twice with 2 x SSC and 0.1% SDS for 10 min each at room temperature, and twice with 0.1 x SSC and 0.1% SDS for 5 min each at 65°C. The hybridized blots were exposed to X-ray films. Northern analyses were repeated three times.

Results

Disease symptoms on the leaves of PI96188. In previous study, genotype PI96188 showed an unusual phenotype against *X. axonopodis* pv. *glycines* strain 8ra (Lee et al., 1997). The first, fully expanded, trifoliate leaves were inoculated with 1×10^8 cfu/ml of *X. axonopodis* pv. *glycines* 8ra. Seven days after inoculation, soybean line PI96188 showed abnormal disease responses distinguishable from the typical symptoms of other tested genotypes. The chlorotic halo that is a typical symptom of bacterial pustule disease was not observed. The susceptible soybean cultivar Jinjoo1 and moderately resistant cultivar Danbaekkong exhibited typical symptoms of bacterial pustule disease. In
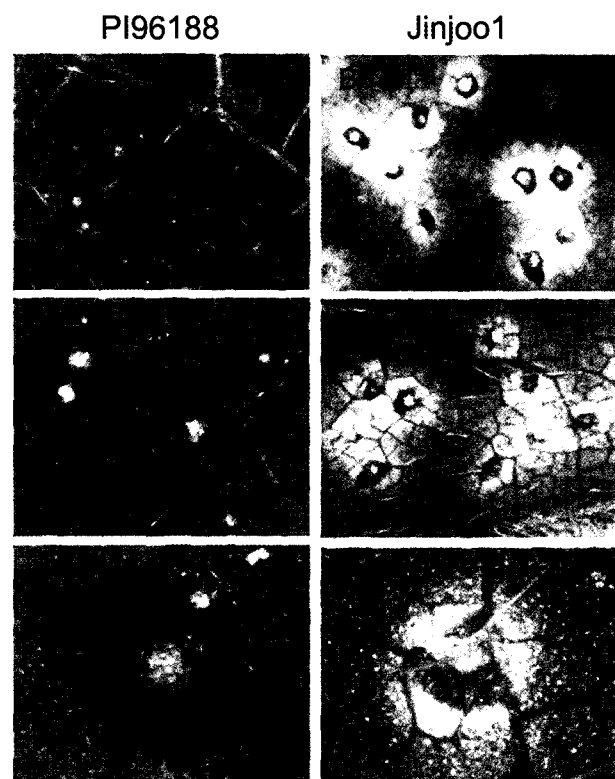

Fig. 1. Disease symptoms on leaves of soybean line PI96188 and cultivar Jinjoo1 infected with *Xanthomonas axonopodis* pv. *glycines* strain 8ra. Fully expanded trifoliolate leaves were photographed at 10 days after inoculation with a bacterial suspension of 10^8 cfu/ml on the abaxial sides (A and B) and the adaxial sides (C, D, E, and F).

the susceptible and moderately resistant cultivars, small pustule symptoms surrounded by chlorotic yellow haloes appeared on the surfaces of infected leaves. Although lesion numbers of line PI96188 is almost similar to those of Jinjoo1, symptoms of the soybean line PI96188 were clearly different from those of the susceptible cultivar Jinjoo1 at 10 days after inoculation (Figs. 1, 2A, B). In the line PI96188, bacteria-induced necroses without chlorotic yellow haloes that is a typical symptoms of bacterial pustule disease occurred in abaxial sides of the leaves, while black pustules were observed.

To determine whether the abnormal necrotic symptoms in the line PI96188 are caused by the different bacterial strains, five bacterial strains, SL1017, SL1018, SL1045, SL1157 and SL2098 of *X. axonopodis* pv. *glycines* that belonged to different races were inoculated on the leaves of the PI96188. Similar characteristic symptoms were observed on the PI96188, irrespective of the bacterial strains inocu-

lated (data not shown). To test whether this novel symptom in PI96188 is caused by other abiotic stress, herbicide 'Basta' known to induce necrosis with yellow haloes was applied on leaves of PI96188 and other soybean cultivars. The response of PI96188 to the diluted Basta was significantly different from that of susceptible cultivars such as Jinjoo1. Basta treatment produced white necrosis surrounded by yellow haloes in the susceptible cultivar Jinjoo1, whereas no yellow haloes were observed in the PI96188 (Fig. 2C, D). Two weeks after the herbicide treatment, the leaves of Jinjoo1 turned completely yellow, although the symptoms on PI96188 remained unchanged. Because Basta is a systemic herbicide, the yellowing regions with necrosis usually spread throughout the whole leaves, eventually causing the cell death. By contrast, in PI96188 treated with Basta, necrosis regions did not spread over the leaves, but the other cultivars including Jinjoo1 and CNS treated with herbicide were defoliated.
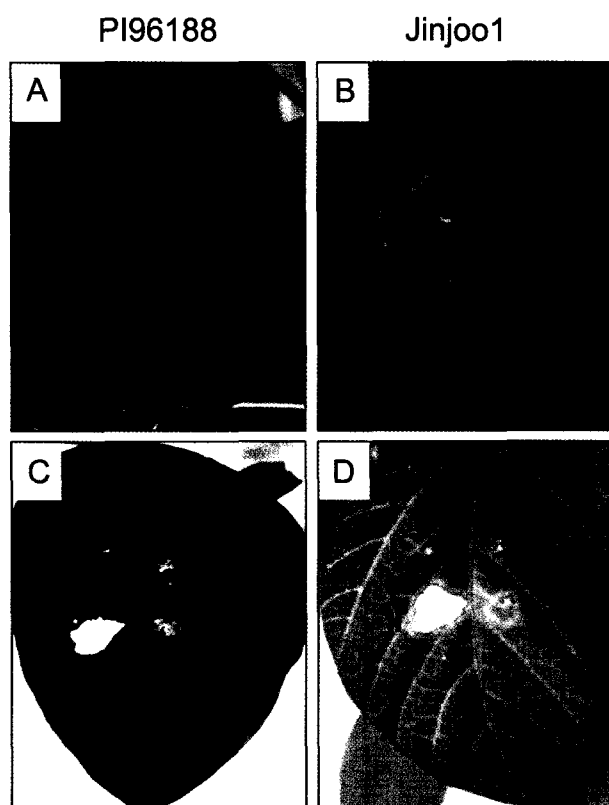
Bacterial growth in PI96188. To determine whether the growth pattern of *X. axonopodis* pv. *glycines* in the soybean line PI96188 is different from those of other soybean cultivars, the growth of *X. axonopodis* pv. *glycines* strain 8ra was examined in leaves of soybean genotypes PI96188, CNS1 and Jinjoo1 (Fig. 3). Fully expanded, trifoliate leaves were inoculated with *X. axonopodis* pv. *glycines* 8ra. During the bacterial infection, disease symptoms were observed and significant differences in bacterial growth patterns were noted between PI96188 and the tested soybean cultivars. Until 6 days after inoculation, we could not observe any significant difference in the bacterial growth in leaves between all the tested soybean genotypes.
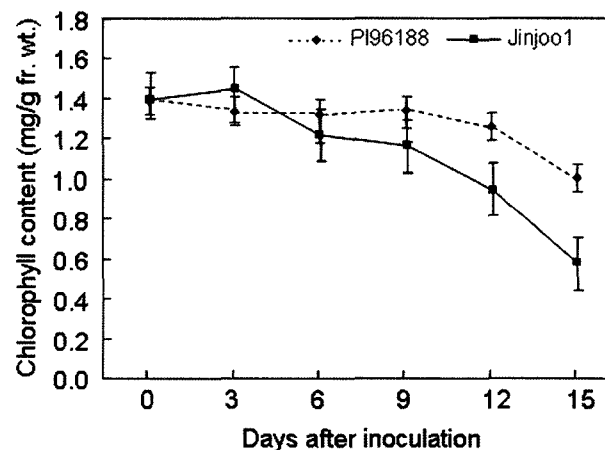

PI96188   **Jinjoo1**

Fig. 2. Differential responses of soybean line PI96188 and cultivar Jinjoo1 to *Xanthomonas axonopodis* pv. *glycines*, *Pseudomonas syringae* pv. *syringae* and the herbicide Basta. Disease symptom development on the leaves of (A) PI96188 and (B) Jinjoo1 at 10 days after infection by *X. axonopodis* pv. *glycines* strain 8ra. Phenotypic changes in (C) PI96188 and (D) Jinjoo1 at 14 days after chemical treatment and bacterial infection. I, 10 mM MgCl₂; II, 0.03% Basta solution; III, *P. syringae* pv. *syringae* strain Pss61; IV, *X. axonopodis* pv. *glycines* strain 8ra.

Fig. 3. Bacterial growth in leaf tissues of soybean line P196188, cultivar CNS (resistant), and cultivar Jinjoo1 (susceptible) infected with *Xanthomonas axonopodis* pv. *glycines* strain 8ra. Fully expanded, trifoliolate leaves were inoculated with a bacterial suspension of 10^8 cfu/ml. Vertical bars represent the means ± standard deviations form three independent experiments.

At 9 days after inoculation, the number of *X. axonopodis* pv. *glycines* 8ra multiplied in PI96188 reached about 10^8 cfu/g fresh weight of soybean leaves. Multiplication of *X. axonopodis* pv. *glycines* 8ra in the susceptible cultivar Jinjoo1 was $10^2 \sim 10^3$ fold higher than that in the resistant cultivar CNS1. In contrast, the bacterial growth in PI96188 was $10^1 \sim 10^2$ fold lower than that in the susceptible cultivar Jinjoo1. The multiplication of *X. axonopodis* pv. *glycines* 8ra in the resistant cultivar CNS1 also was 10-fold higher than that in PI96188 (Fig. 3).

Chlorophyll content in PI96188. Based on the phenotype of PI96188 against *X. axonopodis* pv. *glycines*, we hypothesized that the chloroplasts may remain intact during the development of disease symptoms in PI96188. To test the hypothesis, chlorophyll content was measured in the leaves of the two soybean genotypes (Fig. 4). The chlorophyll contents of PI96188 and Jinjoo1 were 1.4 mg/g fresh weight in uninoculated, healthy leaves, which indicates no significant difference between both soybean genotypes. However, the level of chlorophyll changed dramatically in the susceptible cultivar Jinjoo1 during the *X. axonopodis* pv. *glycines* infection. In the leaves of Jinjoo1, the chlorophyll level drastically declined after infection for 6 days, while that of PI96188 slowly decreased at 9-15 days after inoculation. The initial symptoms on infected leaves appeared at 7-9 days after inoculation. The visible appearance time of initial symptoms coincided with the decrease time in chlorophyll levels. The infected leaves of Jinjoo1 retained only 35% of the chlorophyll in the healthy leaf
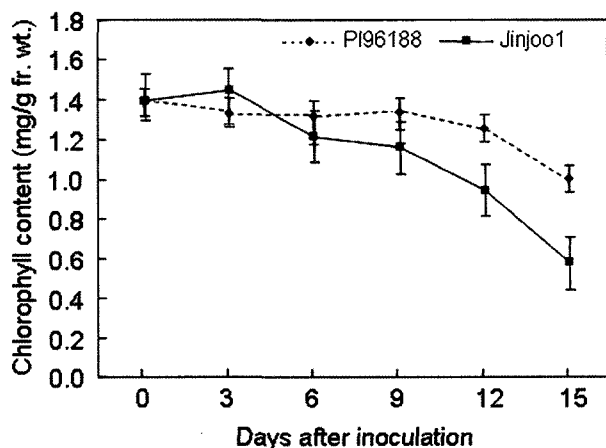
tissue on day 15. By contrast, the chlorophyll level in the PI96188 15 days after inoculation was approximately 70% of the uninoculated, healthy leaves. The Jinjoo1 showed typical symptoms that are small, raised pustules and chlorotic halo, the typical symptoms, which is protruding pustules without chlorotic halo of the PI96188 were observed at 12 days after inoculation.

Ultrastructure of the disease symptoms in PI96188. Since the chlorophyll content in the leaf tissues of PI96188 during the disease development slightly decreased compared to that of the susceptible Jinjoo1 (Fig. 4), we further observed the ultrastructure of chloroplasts around symptoms in leaves of PI96188 and Jinjoo1. Figure 4 shows the electron micrographs of symptoms around pustules in the leaf tissues of PI96188 and Jinjoo1 infected with *X. axonopodis* pv. *glycines* 8ra. Intact grana and few osmio-
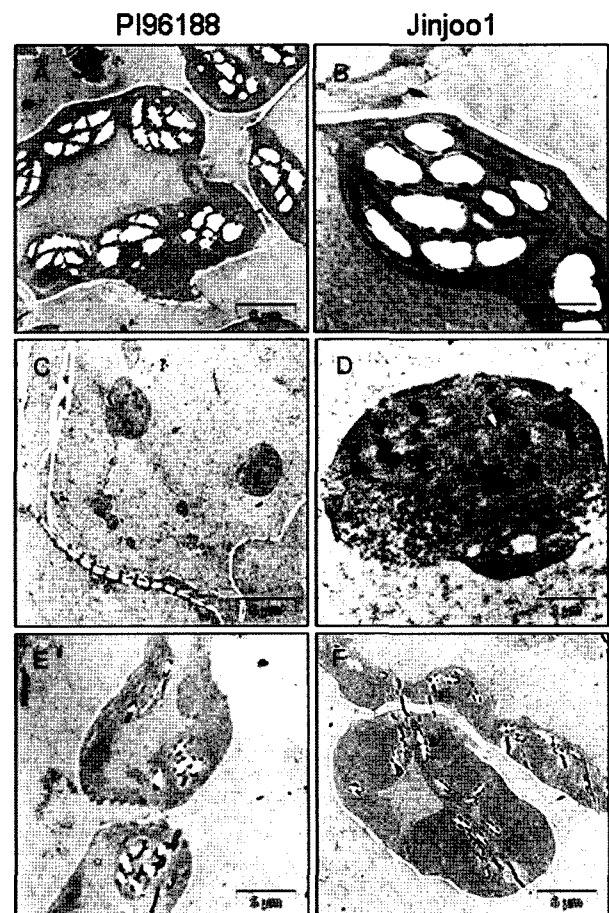

Fig. 4. Chlorophyll contents of leaves of soybean line PI96188 and cultivar Jinjoo1 (susceptible) infected with *Xanthomonas axonoodis* pv. *glycines* strain 8ra. Fully expanded trifoliolate leaves were inoculated with a bacterial suspension of 10^8 cfu/ml. For determination of cholorophyll content, eight leaf discs (8 mm in diameter) were sampled randomly from infected leaves. Vertical bars represent the means ± the standard deviations from three independent experiements.

Fig. 5. Transmission electron micrographs of cross-sections of soybean leaves of soybean line P196188 and cultivar Jinjoo1 (susceptible) at 7 days after inoculation with *Xanthomonas axonopodis* pv. *glycines* strain 8ra. Boundary region of pustule symptoms in (A) PI96188 and (B) Jinjoo1. Chloroplast of a boundary cell in pustule region in (C) PI96188 and (D) Jinjoo1. Uninoculated control of (E) PI96188 and (F) Jinjoo1.

phile starch granules were observed in the chloroplasts in the leaf cells of PI96188. The chloroplasts in the PI96188 were not only intimately attached in a normal size to the cell wall, but also had large starch granules (Fig. 5A, C). Compared to the PI96188, however, mesophyll cells of Jinjool in the infected leaves were enlarged and had few chloroplasts. Chloroplasts were scattered in the mesophyll cells of inoculated Jinjool leaves. The osmiophile granules accumulated in chloroplasts and most of the grana were deteriorated in Jinjool after infection (Fig. 5D). Starch granules were not observed in Jinjool. Chloroplast membranes were destroyed and collapsed (Fig. 5B, D). The mesophyll cells of uninoculated, healthy leaves of Jinjool and PI96188 had a number of normal chloroplasts intimately attached to the cell wall (Fig. 5E, F). They also contained small starch granules and intact grana.

Expression of some senescence-, photosynthesis-, and defense-genes in PI96188. The major differences in disease symptoms between genotype PI96188 and susceptible cultivar Jinjool after inoculation was the absence and presence of chlorosis around the necrotic lesion, respectively, known to be a major symptom of senescence (Fig. 1, Fig. 2). Three groups of soybean genes expressed during senescence were investigated to determine whether the soybean senescence-, photosynthesis- and defense-related

genes play crucial roles for the development of the novel disease symptom on the leaves of soybean genotype PI96188. Ethylene biosynthesis-related genes, such as 1-aminocyclopropan carboxylic acid synthase (ACC synthase, ACS) that function in the rate-limiting step for ethylene biosynthesis are differentially modulated by developmental, hormonal and environmental factors, including mechanical wounding, fungal elicitor, auxin treatment and fruit ripening (Abeles et al., 1992). The expression patterns of the ACS1 and ACS7 genes in the infected leaves of PI96188 were different from those in Jinjool at 1-3 days after inoculation with X. axonopodis pv. glycines (Fig. 6). At 2 days after inoculation, the induction of the ACS1 gene in PI96188 was 2-fold lower than that in Jinjool (Fig. 6A, B). In contrast, the gene expression of ACS7 induced in PI96188 was induced at the 2-fold higher level than that in Jinjool (Fig. 6C, D). A soybean photosynthesis-related gene, termed ribulose-1,5-bisphosphate carboxylase small subunit gene (rbcS), was examined for its expression in the infected leaves of the two soybean genotypes PI96188 and Jinjool. The expression pattern for soybean rbcS gene in PI96188 was obviously different from that in Jinjool (Fig. 7). A strong expression of the rbcS gene in healthy leaves of susceptible cultivar Jinjool rapidly decreased 2 h after inoculation with X. axonopodis pv. glycines and increased at 6 and 12 h, followed by a decrease on day 1 and 2 and an
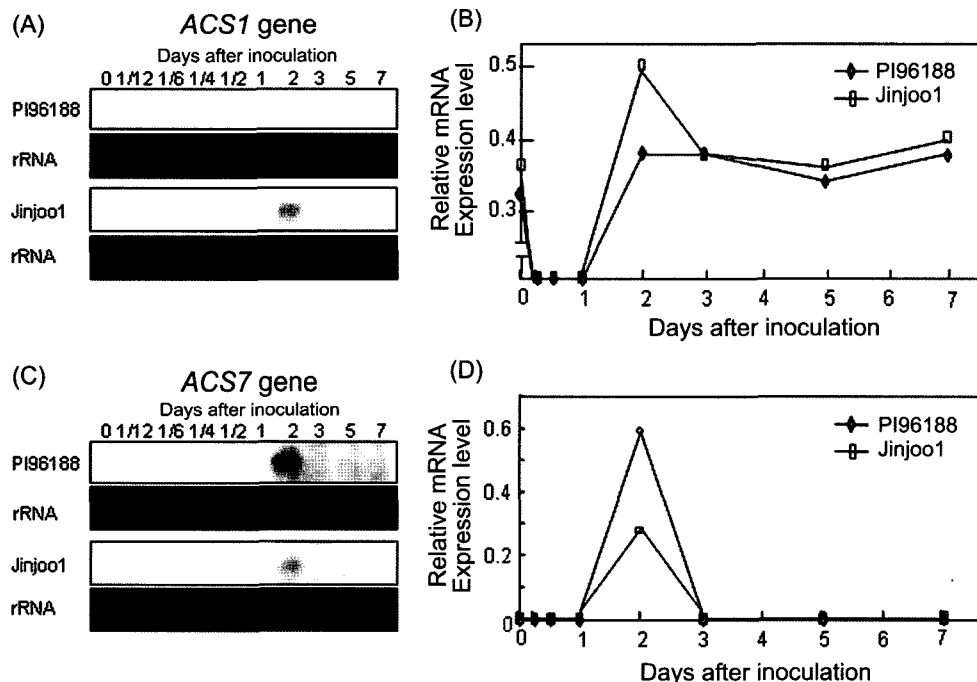

Fig. 6. RNA gel blot analysis of expression of soybean ACS1 gene (a) and ACS7 gene (c) in soybean line PI96188 and cultivar Jinjool (susceptible) infected with Xanthomonas axonopodis pv. glycines. The relative amounts of the mRNA accumulation of ACS1 (b) and ACS7 gene (d) are shown in PI96188 and Jinjool. Total RNAs were isolated from soybean leaves at various time intervals after inoculation. Twenty micrograms of total RNAs from each sample was loaded in each lane. The EcoRI fragment of soybean ACS1 and ACS7 inserts in pGem were ^{32}P-labeled and used as a probe.
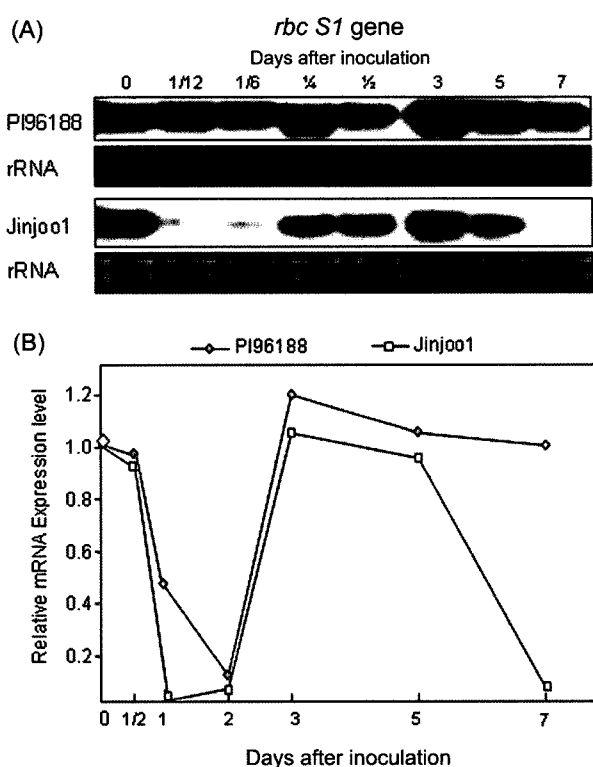
Fig. 7. (A) RNA gel blot analysis of expression of ribulose-1,5-bisphosphate carboxylase small subunit (*rbcS*) gene in soybean line PI96188 and cultivar Jinjoo1 (susceptible) infected with *Xanthomonas axonopodis* pv. *glycines* strain 8ra (B) Relative amounts of mRNA accumulation of the *rbcS* gene at various time intervals after inoculation. Total RNAs were isolated from soybean leaves at various time intervals after inoculation. Twenty micrograms of total RNAs from each sample was loaded in each lane. The *EcoRI* fragments of soybean *rbcS* inserts in pGEM-T was ^{32}P-labeled and used as a probe.

increase on day 3 after inoculation. In contrast, the strong expression of the *rbcS* gene in the PI96188 healthy leaves remained at a high level during the bacterial infection for 7 days, except for 1-2 days of infection. Soybean defense-related genes, such as peroxidase and isoflavone synthase (*IFS*) genes were expressed in a similar manner in the leaves of the two soybean genotypes PI96188 and Jinjoo1 after infection by *X. axonopodis* pv. *glycines* (Fig. 8A, B). Only 1-3 h after bacterial infection, induction of the *IFS* gene was more pronounced in PI96188 than in the susceptible cultivar Jinjoo1.

Discussion

To evaluate the responses of soybean genotypes to bacterial pustule disease caused by *X. axonopodis* pv. *glycines*, soybean cultivars and lines were collected from the soybean-growing areas world-wide. Typical symptoms of bacterial pustule disease usually developed in soybean
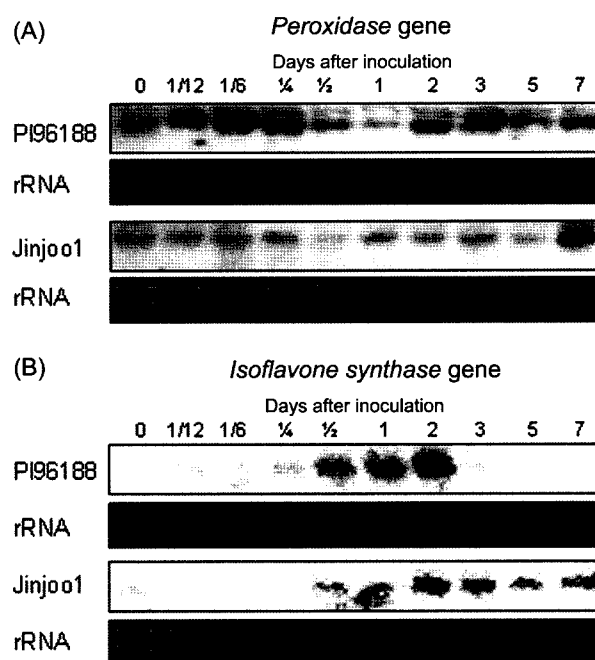

Fig. 8. RNA gel blot analysis of expression of peroxidase (A) and isoflavone synthase gene (B) in soybean line PI96188 and cultiva Jinjoo1 (susceptible) infected with *Xanthomonas axonopodis* pv. *glycines* strain 8ra. Twenty micrograms of total RNAs from each sample was loaded in each lane. The *EcoRI/XbaI* fragments of soybean peroxidase inserts in pBluescript SK+ was ^{32}P labeled and used as a probe.

leaves are small yellow haloes to brown lesions with pustules raised in the lesion center. However, among the 75 genotypes collected, genotype PI96188 showed necrosis without haloes but still had pustules at the background of the necrotic lesions (Fig. 1, Fig. 2). The major difference in the disease symptom between PI96188 and the other soybean cultivars, either susceptible or resistant to the bacterial pustule disease, is that the novel necrotic symptoms appearing on the leaves of PI96188 do not have chlorotic haloes. The disease reaction of PI96188 was not race-specific, because the responses of PI96188 against the five different strains SL1017, SL1018, SL1045, SL1157 and SL2098 of *X. axonopodis* pv. *glycines* were very similar to one another (data not shown). Glutamine can protect plants from chlorosis (Patil and Tam, 1972). Mode of action of Basta is known as the inhibition of glutamine synthethase. To test the effect of chlorosis by glutamine, Basta were treated in soybean plants. Treatment of PI96188 with herbicide Basta usually induced necrosis surrounded by chlorotic regions. Although Basta treatment caused necrosis in the PI96188 leaves, however, there was no yellow chlorosis surrounding the necrotic lesion occurred typically in other soybean cultivars. Chlorophyll content of PI96188 was different from that of cultivar Jinjoo1.

Chlorotic haloes in Jinjool may be, in part, due to the chlorophyll degradation. Together, the data suggest that this typical symptom of PI96188 against pathogens and herbicide might be a unique response. PI96188 may prevent to spread some signals from Basta-treated tissue to adjacent region or has some mechanisms to block chlorosis signal.

In an earlier study, yellowing chlorotic haloes was found to be a macroscopic symptom of chloroplast degradation (Braun, 1955). In the susceptible cultivar Jinjool, meso-phyll cells in the infected leaves were abnormally larger than the healthy control ones, whereas those of PI96188 were not. Moreover, the chloroplasts in the infected leaves of PI96188 had vacuoles and intact grana, which indicates that chloroplasts function normally in this genotype during the bacterial infection. Guiamet and Giannibelli (1994) reported that in parallel with thylakoid degradation, osmio-phile granules, called plastoglobuli, which are deposits of carotenoids, carotenoid esters and free fatty acids derived from lipid breakdown, accumulated in chloroplasts of the yellowing symptom regions in soybean leaves. In the present study, osmiophile granules were observed in the chloroplasts within the boundary of pustule symptoms in Jinjool, whereas osmiophile granules did not accumulate in those of PI96188. These data suggest that delayed or no chlorophyll degradation occurred around the necrosis region of the infected leaves of PI96188.

The pustules were well developed in the PI96188 leaves after infection with X. axonopodis pv. glycines, which sug-gests that this novel necrotic response is basically different from the hypersensitive resistant response usually occurring in the soybean resistant cultivars. To differentiate this PI96188 necrotic response from the hypersensitive response of resistant cultivars, the multiplication of X. axonopodis pv. glycines were compared between PI96188 and resistant cultivar CNS. In general, the bacterial growth in PI96188, Jinjool, and CNS was almost similar until 5 days after inoculation. At 9 days after inoculation with X. axonopodis pv. glycines 8ra, the bacteria multiplied up to 10^8 cfu/g leaves in the PI96188. The number of bacteria in PI96188 was 10 to 100 fold lower than that in the susceptible cultivar Jinjool and 10 fold higher than that in resistant cultivar CNS. Higher bacterial growth in PI96188 than in the resistant cultivar CNS1 strongly suggests that the novel necrotic response of PI96188 with pustule development may be distinguishable from the hypersensitive, resistant response. Soybean genes differentially expressed by patho-gen infection have extensively been studied to better understand functional genomics associated with disease resistance in Glycine max (Jeong et al., 2005A, 2005B). To elucidate the novel necrotic response in PI96188 at the molecular level, we further examined the expression of

genes involved in the leaf senescence, photosynthesis or defense response. Ethylene is a gaseous hormone in plant associated with senescence, fruit ripening, and yellowing symptoms (Stall and Hall, 1984). ACS is the enzyme that catalyzes the conversion of S-adenosylmethionine to ACC, as a precursor of ethylene (Abeles et al., 1992). It had recently been demonstrated that the expression of ACS1 gene was strongly induced during the wounding or senescence, but ACS7 gene was upregulated in mungbean (Kim et al., 2001). In our study, ACS1 gene expression was downregulated, whereas ACS7 gene was significantly induced in PI96188 compared to the susceptible cultivar Jinjool. These results are consistent with the general features of ACS1 and ACS7 gene expression, which support the notion that PI96188 did not show chlorosis around the novel necrotic symptom compared to Jinjool during the bacterial infection. The photosynthesis-related rbcS gene expression was much higher in PI96188 than that in Jinjool during the bacterial infection, which indicated that chlorosis formation inhibited the rbcS gene expression in the susceptible cultivar. However, because PI96188 exhibited a novel necrotic symptom without chlorosis, the rbcS gene expression remained relatively high and stable in PI96188. In contrast, expression of all tested defense-related genes was similar in PI96188 and Jinjool. In general, some defense-related genes, such as isoflavone synthase gene were strongly induced during the resistance reactions, especially including the hypersensitive response. These results suggest that the novel necrotic response in PI96188 may not be directly regulated by defense-related genes in soybean.

This novel necrotic symptom was dissimilar to the hyper-sensitive response, which is known as a localized plant cell death (Hammond-Kosack and Jones, 1997), restricts patho-gen growth at the site of the attempted infection and triggers systemic resistance responses in the non-inoculated cells (Greenberg, 1997) due to the following reasons. First, this atypical necrotic symptom produced a pustule below the necrotic lesion which is a typical symptom of bacterial pustule disease caused by X. axonopodis pv. glycines. Second, this necrotic lesion spread slowly and the pustule grew bigger. However, the hypersensitive response caused by the nonpathogen P. syringae pv. syringae was not continuously enlarged, especially being quite similar to that of the susceptible cultivar Jinjool (Fig. 2C, D). Third, the number of lesions in the infected leaves of PI96188 was almost same as that of the susceptible cultivar Jinjool (data not shown). Fourth, X. axonopodis pv. glycines was able to multiply in PI96188 as fast as in the susceptible cultivar Jinjool compared to the resistant cultivar CNS1 until 6 days after inoculation, while pathogenic bacteria can not multiply in HR (Fig. 3).

The novel necrotic response of PI96188 unique to pathogens and chemicals may be useful to better understand the mechanisms of chlorosis against biotic or abiotic stresses in plants. In most plant diseases, chlorosis reduces photosynthesis, ultimately leading to the damage in the quantity and quality of the crop product. The novel necrotic symptoms observed in PI96188 is predicted to be a resistance response generally effective against *X. axonopodis* pv. *glycines* races, although the bacteria are able to multiply in PI96188. Results of this investigation suggest that the soybean genotype PI96188 may be valuable as a unique genetic source in soybean breeding programs towards the improvement of disease resistance.

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